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## ORIGINAL ARTICLE

# Influence of periodontal treatment on subgingival and salivary microbiotas

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## Abstract

**Background:** The purpose of this study was to characterize and compare subgingival and salivary microbiotas before and after periodontal treatment to learn if any changes of the subgingival microbiota were reflected in saliva. We tested the hypothesis that salivary levels of specific periopathogens correlate with corresponding subgingival levels before and after periodontal treatment.

**Methods:** Twenty-five patients with generalized chronic periodontitis completed the study. Stimulated saliva samples and subgingival plaque samples were collected at baseline and 2, 6, and 12 weeks after nonsurgical periodontal therapy. Subgingival and salivary microbiotas were processed by means of the Human Oral Microbe Next Generation Sequencing (HOMINGS) technique and characterized based on relative abundance. Spearman signed rank test was used to test correlation of periopathogens in subgingival and saliva samples.

**Results:** Periodontal treatment resulted in significantly higher relative abundance of *Streptococcus*, *Rothia* and *Actinomyces* in combination with a significant decrease in *Porphyromonas* and *Treponema* in subgingival plaque samples. Relative abundance of the overall predominant genera in saliva was not influenced by periodontal treatment. However, there was a positive correlation between samples of subgingival plaque and saliva before and after periodontal treatment ( $p < 0.0001$ ) with respect to relative abundance of specific periopathogens, such as *Porphyromonas gingivalis* ( $r = 0.68$ ), *Prevotella intermedia* ( $r = 0.72$ ), *Filifactor alocis* ( $r = 0.58$ ), *Treponema denticola* ( $r = 0.51$ ), *Tannerella forsythia* ( $r = 0.45$ ) and *Parvimonas micra* ( $r = 0.45$ ).

**Conclusions:** Subgingival and salivary abundance of periodontal pathogens correlated before and after treatment. Thus, data from this study suggest that periopathogens identified in saliva may be spill-over from the subgingival microbiota.

## KEYWORDS

16S rRNA, bacteria, microbiology, periodontitis, saliva

Periodontitis is a biofilm-mediated, multifactorial disease, and the subgingival microbiota is critically involved in initiation, maintenance and progression of the disease.<sup>1</sup> The subgingival niche offers ecological conditions with available

nutrients, which favor growth of a diverse microbiota.<sup>2,3</sup> Subgingival abundance of specific periodontal pathogens, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* is considered a major risk factor of



periodontitis,<sup>4</sup> which is why screening for these bacteria in subgingival plaque samples may be relevant in clinical trials.<sup>5,6</sup>

Saliva has been suggested an alternative to local microbial sampling for studies on the oral microbiota, primarily because saliva can be easily and non-invasively collected.<sup>7</sup> Although saliva is sterile when entering the oral cavity,<sup>8</sup> a saliva sample harbors a diverse and individualized microbiota.<sup>9,10</sup> Thus, the salivary microbiota is thought to be composed by bacteria shed from oral surfaces,<sup>11</sup> especially the tongue and throat, which are constantly lubricated by saliva.<sup>12</sup> However, differences in the salivary microbiota have been reported in periodontitis patients compared to orally healthy controls.<sup>13,14</sup> Furthermore, several cross-sectional analyses have shown a positive correlation between subgingival and salivary levels of putative periopathogens such as *P. gingivalis*, *T. forsythia*, and *T. denticola*.<sup>15–18</sup> It is therefore possible that salivary presence of periodontal pathogens may reflect dispersal of these bacteria from the subgingival microbiota into saliva. However, periodontal pathogens are also found as part of the resident microbiota of the tongue<sup>19</sup> and salivary presence of periodontal pathogens has been reported in healthy individuals.<sup>20</sup> Thus, periodontal pathogens identified in saliva might also be dispersed from the tongue microbiota, which is why the origin of periopathogens in saliva remains speculative. Interventional studies with simultaneous characterization of subgingival and salivary microbiotas are therefore needed to reveal whether saliva in fact reflects subgingival levels of specific periopathogens.

Periodontal treatment that is, scaling and root planing, induces ecological changes of the subgingival environment, and cause alterations in the composition of the subgingival microbiota.<sup>21,22</sup> However, to the best of our knowledge the potential perturbation effect of scaling and root planing on the salivary microbiota has so far not been investigated. Thus, the purpose of this study was to characterize and compare subgingival and salivary microbiotas before and after periodontal treatment, to learn whether changes of the subgingival microbiota were reflected in salivary microbiota. We tested the hypothesis that salivary levels of specific periopathogens correlate with corresponding subgingival levels before and after periodontal treatment.

## 1 | METHODS

### 1.1 | Study population and baseline clinical examination

The study was performed from September 2016 through January 2017 at the University of Copenhagen, Department of Odontology. A sample size of  $n = 31$  was calculated using longitudinal data on  $\alpha$ -diversity in saliva samples.<sup>23</sup> Thus, a total

of 35 patients with generalized chronic periodontitis were screened for eligibility, and from these 31 were enrolled in the study. Inclusion criteria: age  $\geq 40$  yrs.,  $> 20$  teeth, Caucasian, periodontitis according to predefined criteria as mentioned below. Exclusion criteria: treatment involving caries, hyposalivation, systemic diseases, and current use of any medication with known effect on periodontitis, use of local or systemic antibiotics within the last 3 months, and professional dental cleaning within the last 3 months. Twenty-five participants (M: 12/ F: 13, mean age: 63 yrs., range: 47–75), including seven current smokers, completed the study (four participants were excluded because of antibiotic treatment during the study and two participants dropped out). The study was approved by the regional ethical committee of the capital region of Denmark (H-16016368), reported to the Danish Data Authority (SUND-2016-58), and registered at clinicaltrials.gov (NCT02913248). All participants signed written informed consent before participation.

### 1.2 | Clinical examination

All clinical examinations were performed by the same clinician (MAG). Caries was registered full-mouth clinically and by use of bite-wing radiographs. Periodontitis was diagnosed based on full-mouth registration of probing pocket depth (PD), clinical attachment level (CAL), plaque index (PI), and bleeding on probing (BOP), which were registered at six sites (disto-facial, mid-facial, mesio-facial, disto-lingual, mid-lingual, and mesio-lingual) of each tooth (third molars excluded). Only subjects with a minimum of four teeth with moderate to severe periodontitis as defined by the American Academy of Periodontology<sup>24</sup> were enrolled.

### 1.3 | Study design and sample collection

In this protocol, patients received nonsurgical periodontal treatment, that is, scaling and root planing, at baseline and they were followed for 12 weeks after treatment. Thorough oral hygiene instruction was also given at baseline and at follow-up visits 2 and 6 weeks after treatment. BOP and PI were registered at baseline, week 2, week 6, and week 12, whereas PD and CAL were recorded at baseline and after 12 weeks. Samples were collected from 8 am to 3 pm and great care was taken to collect samples from each participant at the same time of the day. First, a chewing-stimulated saliva sample was collected as previously described.<sup>25</sup> This was followed by collection of a pooled subgingival sample, collected from four sites with the deepest PD using the curette technique.<sup>26</sup> Subgingival plaque samples were suspended in sterile saline (500  $\mu$ l). All samples were placed on dry ice immediately after collection followed by storage at  $-80^{\circ}\text{C}$  until further analysis.



## 1.4 | DNA extraction

Total DNA was extracted using the MagNA Pure 96 instrument.\* Each sample was evaluated with respect to viscosity. Highly viscous samples were liquefied by treatment with DTT (1,4-dithiothreitol, threo-1,4-Dimercapto-2,3-butanediol<sup>†</sup>) at a final concentration of 0,2% v/w for 30 min at 37°C. This mainly applied to saliva samples.

250 µl of sample (optionally liquefied) was enzymatically digested by adding an equal volume of MagNA Pure Bacteria Lysis Buffer\* supplemented with 5U/sample Zymolyase<sup>‡</sup> and incubated at 37°C for 30–20 min. Finally, 25 µl proteinase K (21,5 IU), recombinant PCR grade\* was added and samples were incubated at 60–65°C for 30–45 min.

A total sample volume of 200 µl was processed on the MagNA Pure 96 instrument using the Pathogen\_Universal\_200 protocol and the MagNA Pure 96 DNA and Viral NA small volume kit.\*

## 1.5 | Next-generation sequencing (HOMINGS)

The Human Oral Microbe Identification using Next Generation Sequencing (HOMINGS) technique was used for microbial analysis.<sup>23,27</sup> The laboratory procedures of HOMINGS follow a method modified from a previously published protocol.<sup>28</sup> Initially, quality control of starting material was performed based on DNA concentration measuring and A260/280 with Nanodrop (one sample failed quality control). Next, PCR-amplification of starting DNA (10–50 ng) using universal primers targeting the V3-V4 region of the 16S genes (F341, R806) and AMPure purification was performed. Generated amplicons from 95 samples were pooled in libraries (100 ng), which were gel-purified and quantified by qPCR before being sequenced using MiSeq.<sup>‡</sup> After quality filtering of generated sequences, including removal of bad reads and chimeric sequences, a total of 15,764,637 reads (approx. 441 bp long) were taxonomically assigned.

## 1.6 | Taxonomic assignment

DNA sequences were taxonomically assigned by use of the customized BLAST program named ProbeSeq for HOMINGS.<sup>11</sup> In ProbeSeq DNA sequences are blasted against 692 unique reference sequences (14–40 bases long), which were developed based on taxonomic information retrieved from the HOMD database.<sup>29</sup> A total of 598 reference sequences are species-specific, whereas the remaining 94 sequences are genus specific. At first, each sequence is blasted against the list of species-specific reference sequences. Sec-

ond, if the DNA sequence is not matched with a species-specific reference sequence, the DNA sequence is subsequently blasted against the list genus-specific reference sequences. Third, if the sequence is not matched with a genus-specific reference sequence it is recorded as unassigned. Consequently, in each sample a proportion of the DNA reads are assigned at species-level (all reads assigned to a species-specific reference sequence) and genus-level (all reads assigned to a species-specific reference sequence + all reads assigned to a genus-species reference sequence), whereas the remaining reads are unassigned. Based on this taxonomic assignment, relative abundance is calculated as the percentage of DNA reads assigned each reference sequence versus the total number of reads in each sample.

## 1.7 | Statistics

All data were checked for normality. Clinical data (PI, BOP, PD and CAL) were compared using a repeated t-test. For these analyses, a p-value < 0.05 was considered statistically significant. Relative abundance was employed for comparison of samples using Kruskal-Wallis and Mann-Whitney test with Benjamini Hochberg correction for multiple dependent analyses.<sup>30</sup> For these analyses, an adjusted p-value of < 0.01 was considered as significant. Spearman signed rank test was used to compute correlation of relative abundance of periodopathogens in subgingival and saliva samples. MeV version 4\_9\_0<sup>31</sup> and Prism 5<sup>§</sup> were used as statistical software.

# 2 | RESULTS

## 2.1 | Clinical data

Full mouth recordings of PD, CAL, BOP (%) and PI (%) and site-specific PD and CAL expressed as mean (range) are presented in Table 1. Conventional periodontal treatment resulted in a decrease in PI and BOP, which remained significant throughout the study period ( $p < 0.001$ ). PI remained stable after 2 weeks, whereas BOP gradually increased from week 2 to week 12. Mean levels of full mouth recordings of PD (baseline: 3.4 versus week 12: 3.0) and CAL (baseline: 4.1 versus week 12: 3.7) as well as site-specific PD (baseline: 6.4 versus week 12: 5.0), and site-specific CAL (baseline: 7.0 versus week 12: 5.7) were significantly decreased at week 12 compared to baseline recordings ( $p < 0.001$ ).

## 2.2 | Next-generation sequencing data

A total of 15.7 M DNA reads were generated from 199 microbial samples (subgingival plaque:  $n = 99$ , saliva:  $n = 100$ ),

\* Roche, Mannheim, Germany.

<sup>†</sup> Sigma-Aldrich, Darmstadt, Germany.

<sup>‡</sup> Graphpad, La Jolla, CA, USA.

<sup>§</sup> Illumina, San Diego, CA, USA.

**TABLE 1** Full-mouth and sample site periodontal parameters

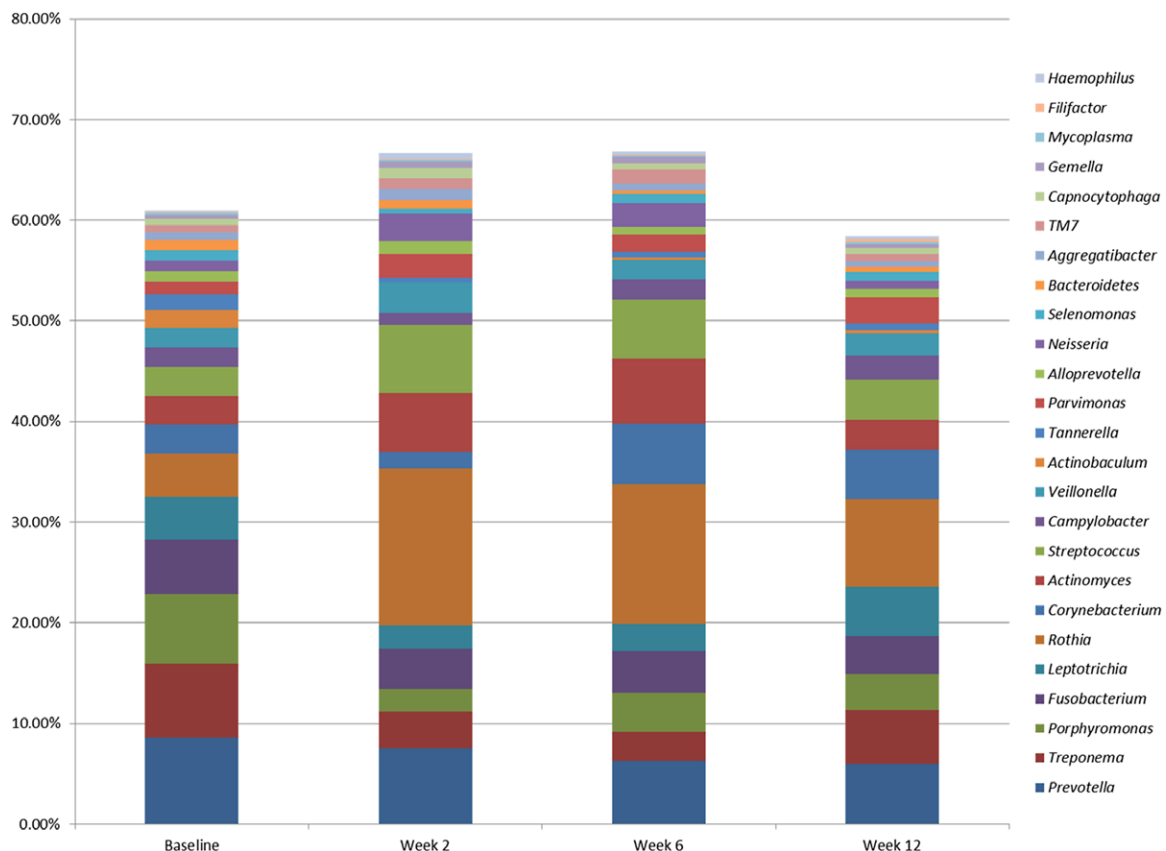
Parameter	Baseline	Week 2	Week 6	Week 12	P-value
PD	3.4 (2.4–5.1) 6.4 (5.0–8.8) <sup>a</sup>			3.0 (2.0–4.4) 5.0 (3.3–7.3) <sup>a</sup>	$P < 0.001$
CAL	4.1 (2.8–6.0) 7.0 (5.5–9.3) <sup>a</sup>			3.7 (2.6–5.6) 5.7 (3.3–8.8) <sup>a</sup>	$P < 0.001$
BOP	56.0 (31.3–97.5)	27.1 (8.7–52.1)	34.6 (10.3–56.8)	41.3 (17.3–74.0)	$P < 0.001$
PI	84.2 (63.0–99.0)	41.0 (7.3–79.1)	43.0 (12.7–75.4)	41.8 (13.8–73.5)	$P < 0.001$

<sup>a</sup>Site-specific recording from pockets sampled for microbial analysis.

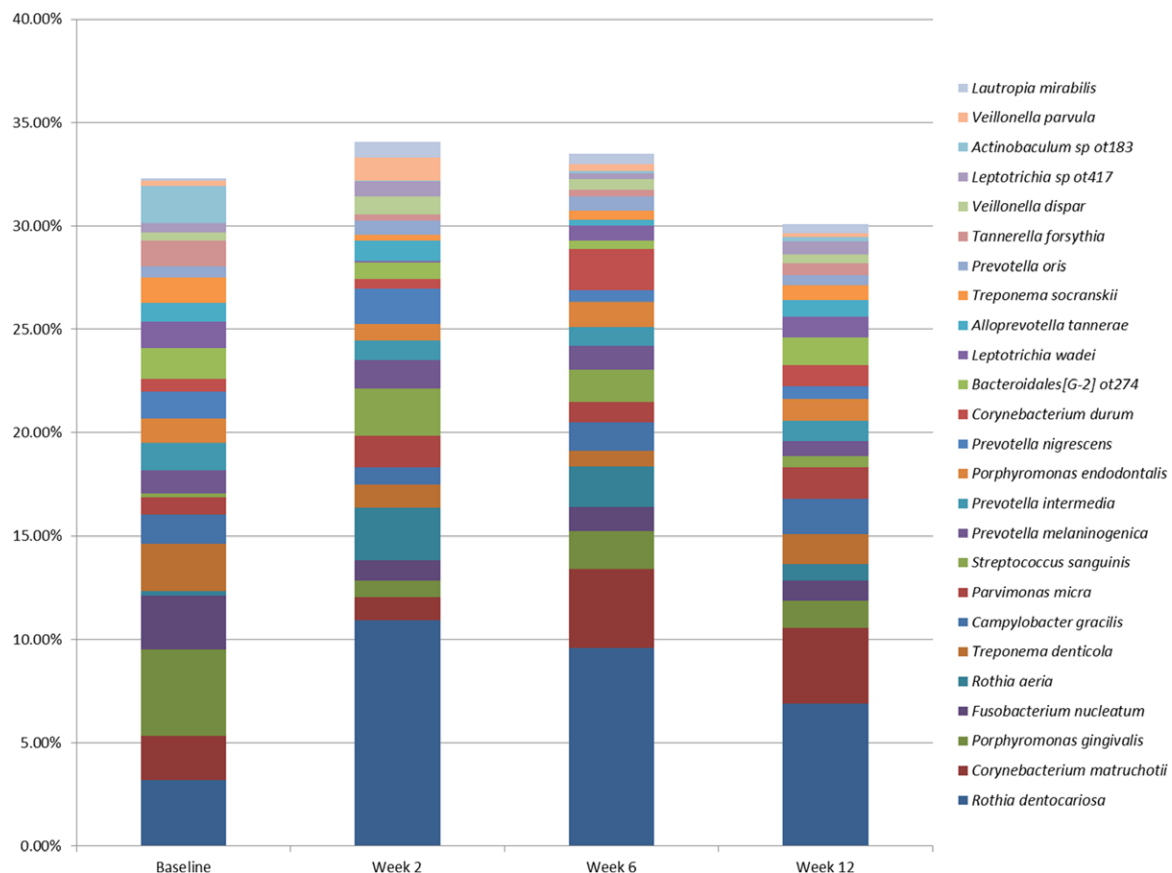
with a mean of 73,763 (range: 31,267–170,022) DNA reads per sample. The mean percentage of reads assigned at genus levels was 70.7% (range: 36.7%–94.2%), whereas the mean percentage of reads assigned at species level was 51.3% (27.9%–82.8%). Consequently, the mean percentage of unassigned reads was 29.3% (range: 5.8%–63.33%). Assignment of DNA reads collectively yielded identification of 507 different bacterial species, with a mean of 185 (range: 75–328) bacterial species per sample. A significantly higher mean number of bacterial species was recorded in subgingival plaque samples ( $n = 198$ , range: 117–328) versus saliva ( $n = 172$ , range: 75–276) ( $p < 0.05$ ).

## 2.3 | Major impact of periodontal treatment on the predominant subgingival microbiota

Relative abundance of the 25 predominant bacterial genera and the 25 bacterial species before and after nonsurgical periodontal treatment is presented in Figures 1 and 2. Periodontal treatment resulted in significantly higher relative abundance of *Streptococcus*, *Rothia*, and *Actinomyces* species in combination with a significant decrease in *Porphyromonas* and *Treponema* species (Figure 1). Specifically, a 5-fold reduction in mean abundance of *P. gingivalis* (4.2% versus 0.8%), a 4-fold decrease of *T. forsythia* (1.3% versus 0.3%), and a 2-fold decrease in *T. denticola* (2.3% versus 1.1%) in

**FIGURE 1** Predominant bacterial genera in subgingival plaque. Mean levels of relative abundance of the 25 predominant genera in subgingival samples at baseline and 2, 6, and 12 weeks after treatment





**FIGURE 2** Predominant bacterial species in subgingival plaque. Mean levels of relative abundance of the 25 predominant species in subgingival samples at baseline and 2, 6, and 12 weeks after treatment

combination with a 10-fold increase in abundance of *Rothia aeria* (0.2% versus 2.6%) and a 3-fold increase in *Rothia dentocariosa* (3.2% versus 10.9%) were recorded two weeks after periodontal treatment (Figure 2). These changes were gradually reversed until 12 weeks after treatment. In addition, periodontal therapy had an impact on microbial diversity of the subgingival niche as  $\alpha$ -diversity at baseline (2.73) was decreased after 2 weeks (2.50) and after 6 weeks (2.60) and completely reversed after 12 weeks (2.78) ( $p = 0.007$ ).

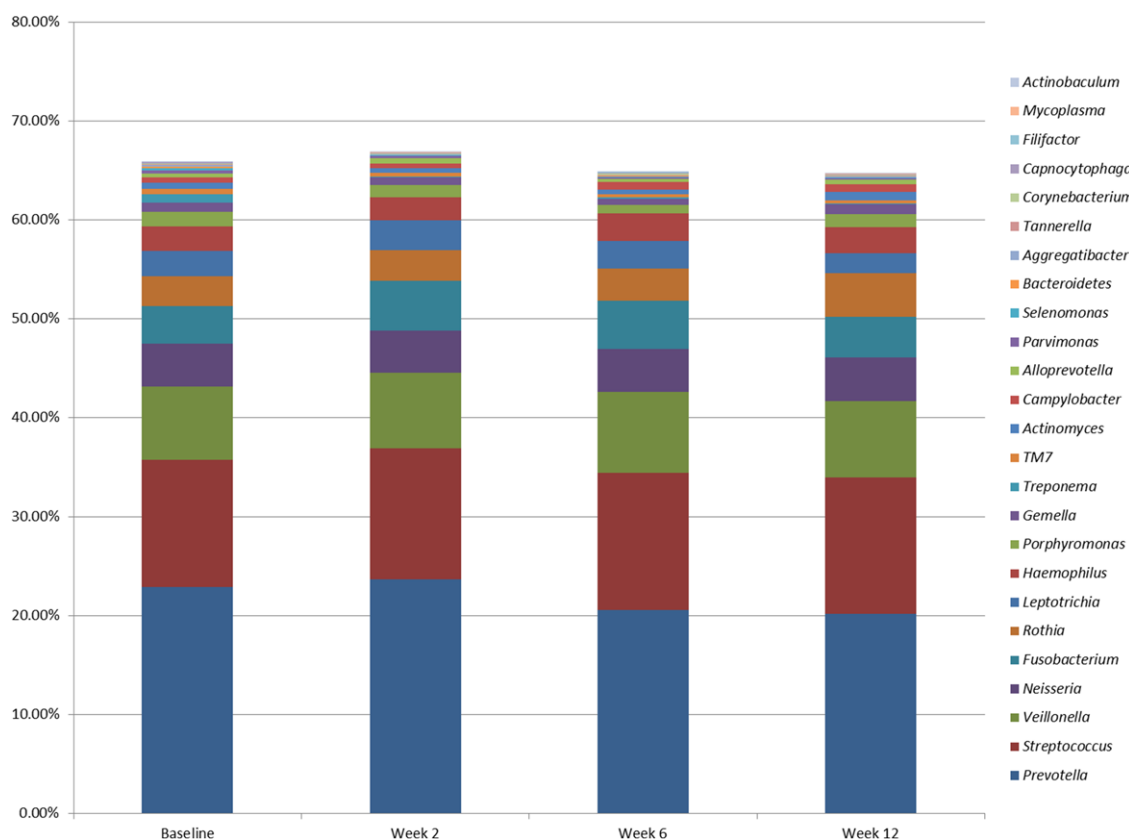
## 2.4 | Minor impact of periodontal treatment on the predominant salivary microbiota

Relative abundance of the 25 predominant bacterial genera and the 25 bacterial species in saliva before and after non-surgical periodontal treatment is presented in Figures 3 and 4. The predominant bacterial genera in saliva were *Prevotella* and *Streptococcus*, which constituted approx. Thirty-five percent of all DNA reads before and after nonsurgical periodontal therapy (Figure 3). In addition, no significant differences in relative abundance of predominant bacterial species were recorded during the 12 weeks follow up period (Figure 4). However, a significant impact of periodontal therapy on  $\alpha$ -diversity was recorded as microbial diversity

recorded at baseline (2.34) was decreased after 2 weeks (2.24) and after 6 weeks (2.21) but completely reversed after 12 weeks (2.35) ( $p = 0.01$ ).

## 2.5 | Correlation between subgingival and salivary abundance of periopathogens before and after periodontal treatment

Correlations between subgingival and salivary abundance of *P. gingivalis*, *P. intermedia*, *T. forsythia*, *T. denticola*, *Filifactor alocis*, and *Parvimonas micra* are presented in Table 2. Comparison of relative abundance in subgingival plaque ( $n = 99$ ) and saliva samples ( $n = 99$ ) collected before and after periodontal treatment showed an overall good correlation of *P. gingivalis* ( $r = 0.68$ ,  $p < 0.0001$ ) and *P. intermedia* ( $r = 0.72$ ,  $p < 0.0001$ ) and a moderate correlation of *F. alocis* ( $r = 0.58$ ,  $p < 0.0001$ ), *T. denticola* ( $r = 0.51$ ,  $p < 0.0001$ ), *T. forsythia* ( $r = 0.45$ ,  $p < 0.0001$ ), and *P. micra* ( $r = 0.45$ ,  $p < 0.0001$ ). Periodontal treatment had a significant impact on correlations between relative abundance in subgingival plaque and saliva, as an increase in correlation of all six periopathogens was recorded at week 2 compared to baseline levels, which gradually decreased during the 12 weeks follow-up period.



**FIGURE 3** Predominant bacterial genera in saliva. Mean levels of relative abundance of the 25 predominant genera in saliva samples at baseline and 2, 6, and 12 weeks after treatment

### 3 | DISCUSSION

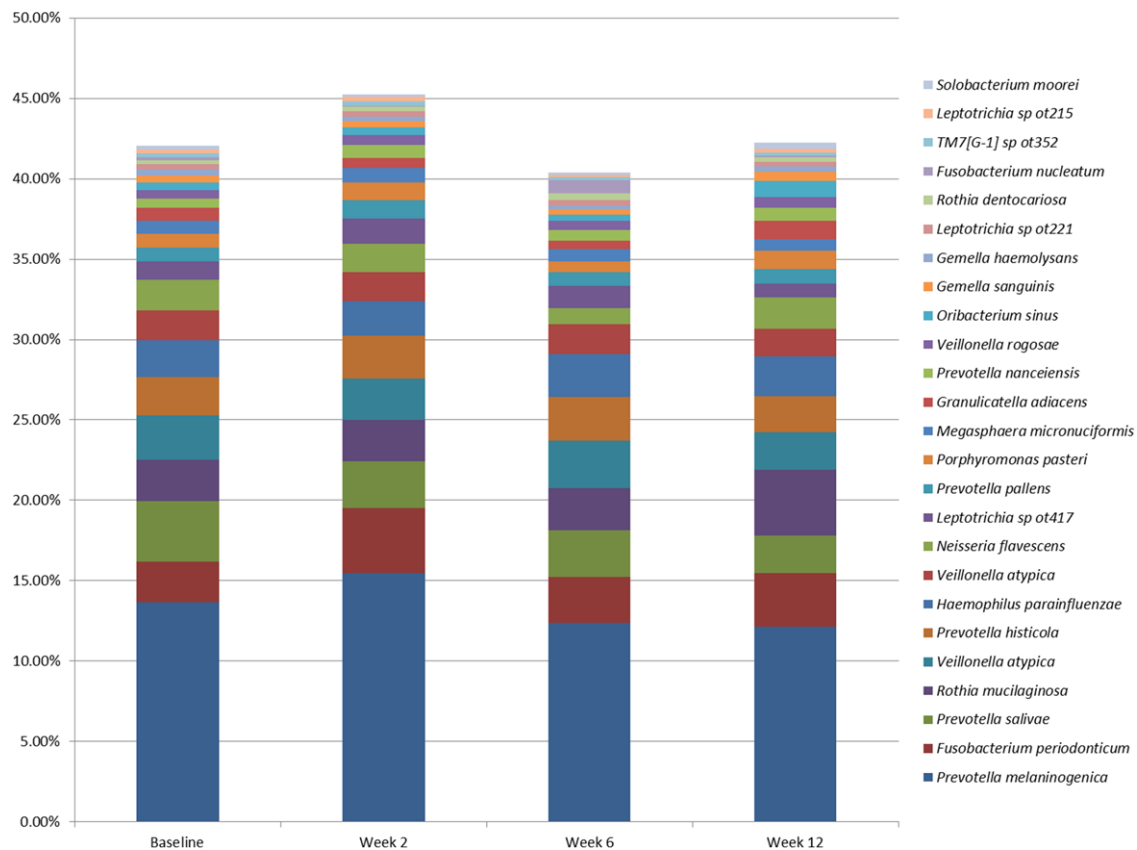
The main finding of the present study was correlation between subgingival and salivary abundance of periopathogens before and after periodontal treatment. To the best of our knowledge, this is the first interventional study to successfully demonstrate an impact of periodontal treatment on salivary levels of specific periopathogens, which correlated with subgingival abundance, in patients with chronic periodontitis.

Some limitations apply to this investigation, including the drop-out of six patients. For practical and economic reasons, analysis of a pooled subgingival sample from the four deepest periodontal lesions was used. However, as all participants had more than the four diseased sites from where samples were taken, information on the complete subgingival microbiota was not obtained. This limitation highlights the major dilemma of using local microbial sampling in clinical periodontology, namely that ideally single-site sampling analysis should be performed.<sup>1</sup> However, this may not always be practically feasible.

Primarily because of the ease and inexpensive nature of saliva sampling and analysis, saliva has been suggested as a substitute to local microbial sampling for monitoring of the oral microbiota.<sup>7</sup> Differences in salivary bacterial profiles have been reported in patients with periodontitis

compared to orally healthy controls,<sup>13,14</sup> and several cross-sectional studies have shown correlation between subgingival and salivary levels of specific periopathogens in periodontitis patients.<sup>15–18</sup> However, red complex bacteria, that is, *P. gingivalis*, *T. denticola*, and *T. forsythia*, are also part of the tongue resident microbiota in orally healthy individuals, which is why salivary identification of periopathogens may not only reflect subgingival presence. A way to elucidate this aspect is to induce a perturbation of the subgingival microbiota and longitudinally record whether changes of the subgingival microbiota are mirrored in the salivary microbiota. Thus, in this study nonsurgical periodontal treatment was performed to perturbate the subgingival microbiota and to reveal the effect in the composition of the subgingival and salivary microbiotas as recorded during a 12 week follow-up period.

As expected, periodontal treatment had an immediate generalized impact on clinical parameters, and the effect of the treatment was comparable with data presented in a systematic review on the efficacy of non-surgical periodontal therapy.<sup>32</sup> Clinical changes were accompanied by alterations in the composition of the subgingival microbiota with a significant decrease in relative abundance of red complex bacteria, that is, *P. gingivalis* (4.2% versus 0.8%), *T. denticola* (2.3% versus 1.1%), and *T. forsythia* (1.3% versus 0.3%) (Figure 2), which is in accordance with previous reports.<sup>21,22</sup> This presumably



**FIGURE 4** Predominant bacterial species in saliva. Mean levels of relative abundance of the 25 predominant species in saliva samples at baseline and 2, 6, and 12 weeks after treatment

reflects ecological changes in the subgingival niche associated with periodontal treatment<sup>1</sup> confirming the intended perturbation effect of non-surgical periodontal treatment on the subgingival microbiota.

To test the impact of periodontal treatment on the core salivary microbiota, relative abundance of predominant bacterial genera and species were compared before and after treatment. Data clearly showed that relative abundance of the core salivary microbiota was merely not influenced by periodontal therapy (Figures 3 and 4). This suggests that *Streptococcus* and *Prevotella* species, which make up the bulk of the salivary microbiota, are primarily dispersed to saliva from other oral surfaces than the subgingival niche. This finding complies with data from another study, which reported that the major contributors to the salivary microbiota are bacteria shed from the tongue, the tonsils and the pharynx.<sup>12</sup>

Periodontitis is considered a poly-microbial disease.<sup>2</sup> However, some specific bacterial species, such as *P. gingivalis*, *T. Forsythia*, *T. denticola*, *P. intermedia* are proposed as putative periopathogens.<sup>4</sup> Because of the advent of contemporary molecular techniques, additional putative periodontal pathogens, including *P. micra* and *F. alocis* have been identified.<sup>33,34</sup> To test the effect of periodontal treatment on salivary abundance of these periopathogens, we performed correlation analysis between salivary and subgingival lev-

**TABLE 2** Spearman rank correlation (*r*) among subgingival and salivary abundance of periopathogens

Species	Baseline	Week 2	Week 6	Week 12	P-value
<i>P. gingivalis</i>	0.60	0.72	0.77	0.71	p < 0.0001
<i>P. intermedia</i>	0.78	0.79	0.72	0.41	P < 0.0001
<i>T. forsythia</i>	0.08	0.56	0.37	0.30	P < 0.0001
<i>T. denticola</i>	0.44	0.49	0.41	0.23	P < 0.0001
<i>F. alocis</i>	0.48	0.62	0.58	0.24	P < 0.0001
<i>P. micra</i>	0.25	0.61	0.34	0.37	P < 0.0001

els of *P. gingivalis*, *T. Forsythia*, *T. denticola*, *P. intermedia*, *P. micra*, and *F. alocis*. Significant correlation on salivary and subgingival levels of *P. gingivalis* ( $r = 0.60$ ) and *P. intermedia* ( $r = 0.78$ ) was recorded at baseline, which agrees with previous cross-sectional analysis, employing various contemporary methods for bacterial identification.<sup>15–18</sup> Further, data demonstrated that salivary levels of periopathogens reflected subgingival alterations as an increase in correlation was noted from baseline to week 2 after periodontal treatment for all six periopathogens (Table 2). In addition, a significant decrease in bacterial  $\alpha$ -diversity in saliva was noted after periodontal treatment. Thus, data from this study suggest that periopathogens identified in saliva presumably are spill-over from the subgingival microbiota.





## 4 | CONCLUSION

In conclusion, subgingival and salivary relative abundance of putative periodontal pathogens correlated before and after periodontal treatment. Therefore, data from this study support the assumption that salivary levels of putative specific periodontal pathogens reflect subgingival colonization.

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## Data availability statement

Access to all data, including DNA sequences, will be granted upon request (dbel@sund.ku.dk).